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Determination of Bisphenol Analogues in Infant Formula Products from India and Evaluating the Health Risk in Infants Asssociated with Their Exposure

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ABSTRACT: Bisphenol A (BPA) is a well-recognized endocrine disruptor, and considering its adverse effects its use in infant bottles has been banned in many countries. Growing concern on the use of BPA has led to its replacement with its analogues in numerous applications. Present is the first report determining the occurrence of seven bisphenols (BPs: BPA, BPAF, BPC, BPE, BPFL, BPS, and BPZ) in Indian infant formula. A reliable and efficient UPLC-MS/MS method for their simultaneous determination was developed and validated in powdered infant formula (n = 68). The limit of quantification of the method was 0.19 ng/g for BPA, BPAF, BPE, BPS and BPZ and 0.78 ng/g for BPC and BPFL. The highest concentration was detected for BPA (mean = 5.46 ng/g) followed by BPZ and BPS. BPAF, BPFL, BPC and BPE were detected in none of the samples. The estimated daily intake (EDI) of total BPs in infants (0−12 months old infants) was determined to be 54.33-213.36 ng/kg b.w./day. BPA mainly contributed to the total intake (EDI = 92.76 ng/kg b.w./day). The dietary exposure to total BPs evaluated in the present study was approximately 1 order of magnitude lower than the reference value of BPA set by EFSA (4 μg/kg b.w./day) and, thus, may not pose considerable risks to infants.

KEYWORDS: *bisphenol analogues, estimated daily intake, India, powdered infant formula, occurrence, health risk assessment*

INTRODUCTION

Endocrine disruptors (EDs) have acquired worldwide attention over the past years owing to their negative health effects on humans and wildlife. EDs are so-called as they are known to mimic the endogenous steroid hormones or their metabolites and intervene in their synthesis, secretion, transport, activity, or disposition.[1,2] Bisphenol A (BPA), a well-known ED, is extensively used in the manufacture of plastics food containers such as infant feeding bottles.[3,4] Being an ED, BPA has been associated with various human diseases, such as diabetes, obesity and cardiovascular and reproductive disorders.[5−7] Growing public concern and strict regulations posed on the use of BPA has led to its replacement with other bisphenol analogues such as bisphenol AF (BPAF) bisphenol C (BPC), bisphenol E (BPE), bisphenol FL (BPFL), bisphenol S (BPS), and bisphenol Z (BPZ) in numerous applications.[8−10] The presence of these analogues in the environment, food or food packages, consumer products, and human samples has been well documented.[10−17] However, owing to the structural similarity to BPA, bisphenol analogues have demonstrated similar or even higher toxic potential than that of BPA.[10,18,19] Thus, structural analogues of BPA might not be suitable and safe alternatives for food and packaging materials, especially intended for infants and children. In this direction, a research program has been promoted by the EU (HBM4 EU) to include other bisphenol analogues such as BPS in the list of prioritized substances to be estimated in biomonitoring studies.[20]

Irrefutably, infants are at the highest risk, even at low dose exposure of BPA.[21] A study by the National Toxicology Program (NTP) and U.S. FDA has indicated the probable effects of BPA on the development of the brain; prostate glands; and behavior in fetuses, infants, and young children.[22] The predominant source of BPA exposure to infants originates from the migration from the lining of the cans into infant formula and from polycarbonate baby bottles.[23,24] Thus, the use of BPA in baby bottles has been banned in European countries and Canada. European Food Safety Authority (EFSA) has fixed the Tolerable Daily Intake (TDI) of BPA at 4 μg/kg b.w./day due to its risk toward human health.[25] In 2011, the European guideline established the specific migration limits (SML) of BPA from plastics in contact with food as 0.6 mg/kg.[26] New regulations lowered the SML value to a more restrictive value of 50 μg/kg, whereas migration of BPA is not permitted from varnishes or coatings applied to materials and articles intended especially for infants and children (<3 years old).[27]

The occurrence of BPA in infant formula has previously been reported from various countries such as Italy,[28] Germany,[29] U.S.,[30,31] U.K.,[32] and China;[33] however, there exists a wide variation in BPA contamination and concentration.[34] To the best of our knowledge, information on the

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exposure of infants to bisphenols (BPs) such as BPA, BPAF, BPC, BPE, BPFL, BPS, and BPZ through infant formula from India is not available yet. Analysis of bisphenols (BPs) requires the development and application of an efficient, sensitive, and reproducible analytical method for accurate risk assessment of BPs exposure. Reports documenting the extraction of BPs using various techniques are available such as solid-phase extraction (SPE), liquid−liquid extraction (LLE), and solid-phase microextraction (SPME).[35−37] However, these methods suffer from pitfalls such as time-consuming, tedious, expensive, and requiring high cleanup step.[38] Therefore, we aim to develop a sensitive and efficient LC-MS/MS method for the quantification of bisphenol analogues (BPA, BPAF, BPC, BPE, BPFL, BPS, and BPZ) in powdered infant formula from different Indian brands. Sample treatment was based on QuEChERS technique which was employed to eliminate the unwanted substances like sugars, fats, lipids, and proteins followed by a cleanup step to obtain high extraction efficiency and sensitivity.[39−41] Based on the measured concentration of BPs in infant formula and recommended daily consumption rate, their estimated daily intake (EDI) in the male and female infant population was calculated. As the data on limits of acceptability is available only for BPA, information obtained from the present study would help assess possible exposure of BPA as well as other bisphenol analogues (BPAF, BPC, BPE, BPFL, BPS, and BPZ) to infants from infant formula, thus, reducing the knowledge gap in this field.

MATERIAL AND METHODS

Reagents and Chemicals. Bisphenol analogues such as BPA (purity ≥99%), BPAF (purity ≥99.0%), BPC (purity ≥99.0%), BPE (purity ≥98.0%), BPFL (purity ≥99.0%), BPS (purity ≥98.0%), and BPZ (purity ≥99.0%) were purchased from Sigma-Aldrich (Bangaluru, India). Acetonitrile (ACN), methanol (MeOH), ammonium solution, ammonium formate, ammonium acetate, and formic acid of LC-MS grade were procured from Fischer Scientific (Mumbai, India). Bisphenol A-d10 (IS-A), bisphenol AF-d4 (IS-AF), bisphenol E-d12 (IS-E), and bisphenol-S d8 (IS-S) were used as internal standard (IS) and procured from Toronto Research Chemicals (TRC, Toronto, Canada). Milli-Q water was provided by Integral Water Purification System Merk Millipore (Millipore India Pvt Ltd., New Delhi, India). Multi-Screen Solvinert Filter Plate (96-well device with 0.45 μm PTFE membranes) was obtained from Millipore Corporation (Millford, U.S.A.). QuEChERS containing anhydrous magnesium sulfate ($MgSO_4$; 99.5% purity) and sodium chloride (NaCl) were purchased from Merck Life Sciences (Mumbai, India); the sorbent primary secondary amine (PSA; particle size 40 μm) from Agilent (US, U.S.A.) and Phenonemex Kinetex C18 (100 x2.1 mm, 1.7 μm) column was purchased from Phenomenex India (Hyderabad, India).

Sample Collection. A total of 68 milk-based powdered infant formula samples (recommended for infant aged 0−12 months) belonging to different brands were purchased from Lucknow city of India between November 2018 and January 2019. The sample containers having different packaging types such as plastic pack (n = 12), paper carton (n = 24), and metal can (n = 32) were selected. All collected samples were stored at room temperature (25 ± 5 °C) before analysis.

Preparation of Stock Solution and Standard Solution. The standard stock solutions of the individual bisphenols and IS were prepared in acetonitrile (1 mg/mL) and were stored at −20 °C. A ten-point calibration curve was made by spiking the appropriate volume of stock solution in blank formula matrix to obtain the concentration range of 0.19−100 ng/g for BPA, BPAF, BPE, BPS, and BPZ and 0.78−100 ng/g for BPC and BPFL. For method validation, quality control (QC) samples were prepared in the same way at two concentration levels, i.e., 0.19 ng/g (limit of quantification, LOQ) and 80 ng/g (high quality control, HQC) for BPA, BPAF, BPE, BPS, and BPZ and 0.78 and 80 ng/g for BPC and BPFL. The stock solutions were finally stored at −20 ± 2 °C until use.

Sample Preparation. Sample preparation consisted of the following steps: 5 g of the powdered formula was weighed and a mixture of BPs was spiked into it. Subsequently, 5 mL of Milli-Q water was added, and the mixture was shaken well by hand. After ensuring that the formula is completely dissolved in Milli-Q water, 5 mL of ACN containing IS (5 ng/mL) was added into the mixture. Next, 2 g of anhydrous magnesium sulfate was added and slightly vortexed and then 1 g of sodium chloride was added. Afterward, the tube was shaken on a roto spin cyclomixer (Spinix Tarsons, Kolkata, India) for 10 min and sonicated for another 10 min. The mixture was then centrifuged at 5000 rpm for 10 min (Eppendorf, Hamburg, Germany), and the supernatant was transferred in a 15 mL tube. The supernatant was frozen at −20 °C ± 5 °C for 30 min and finally centrifuged at 5000 rpm for 10 min. The upper layer was transferred into the Solvinert filter plates to remove the unwanted endogenous compound, and the plate was centrifuged at 4000 rpm for 2 min. An aliquant (100 μL) was transferred into two vials, one having ammonia solution (0.4 μL from 25% ammonia solution; final concentration 0.1% ammonia in the sample) for BPA, BPE, BPFL, BPAF, BPC, and BPZ and another one without ammonia solution for BPS. The vials were tightly capped and vortexed for 20 s and finally submitted for UPLC-MS/MS analysis.

UPLC-MS/MS Analysis. UPLC system (Sciex, Canada) was used to inject an aliquot (10 μL) of the processed samples on Phenonemex Kinetex C18 (100 × 2.1 mm, 1.7 μm) column. Before use, mobile phases were filtered through a 0.22-μm Millipore filter and ultrasonically degassed for 2 min. Acetonitrile (A) and Milli-Q water (B) were chosen as the mobile phase and delivered at a flow rate of 0.6 mL/min in a gradient mode (0−0.5 min 95% B; 0.5−4.0 min 5% B; 4−5.9 min 5% B; 5.9−6.0 min 95% B, and 6.0−6.5 min 95% B). Mass spectrometric detections were carried out on a 5500 QTRAP MS/MS (Sciex, Canada) in negative ionization mode. The specific values for parameters related to the analytes are shown in Table 1. Analyst software (version 1.7) was used for data acquisition and quantification.

Table 1. Tandem Mass Spectrometer Parameters Used to Analyze BPs[a]

analyte		MRM transition	CE (eV)	DP (eV)	CXP (eV)
bisphenol-A	quantifier	227.4→133.0	−30	−75	−17
	qualifier	227.4→211.1	−25		
bisphenol-AF	quantifier	355.1→265.0	−35	−120	−16
	qualifier	355.1→161.0	−39		
bisphenol-C	quantifier	255.2→238.9	−26	−60	−16
	qualifier	255.2→156.0	−30		
bisphenol-E	quantifier	213.0→197.0	−25	−60	−16
	qualifier	213.0→148.0	−30		
bisphenol-FL	quantifier	349.2→256.2	−35	−90	−16
	qualifier	349.2→192.0	−35		
bisphenol-S	quantifier	242.0→183.8	−40	−80	−15
	qualifier	242.0→104.9	−39		
bisphenol-Z	quantifier	267.1→173.0	−39	−70	−16
	qualifier	267.1→222.9	−39		
IS-A		233.1→138.0	−33	−120	−16
IS-E		225.1→125.9	−37	−50	−16
IS-S		257.0→111.9	−30	−70	−17
IS-AF		339.1→269.0	−30	−80	−16

[a]Abbreviations: MRM, Multiple reaction monitoring; DP, declustering potential; CE, collision energy; CXP, collision exit potential; EP, entrance potential; CUR, curtain gas; CAD, collision gas; GS1, nebulizer gas; GS2, turbo ion gas; IS, internal standard.

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Method Validation. The validation of the developed analytical method was performed as per the SANTE Guidance Document in term of linearity, accuracy, precision, recovery and matrix effect.[42,43] Also, LOD and LOQ of the method were determined.

As BPs are ubiquitous in the environment, the formula samples collected from the market were screened for the presence of BPs and those which showed no signals of BPs were used as blank matrices for preparing the calibration curve and validation samples. Each validation batch consisted of a solvent blank, procedural blank, zero sample (matrix processed with only IS), ten calibration standards, two sets of quality control (QC) samples. Calibration curves consisting of standard samples were made by plotting the peak area ratio of analyte to IS versus the actual concentration of calibration standards. Linear regression analysis and a weighting factor of $1/x^2$ was used to determine the slope, intercept and correlation coefficient. The acceptability criteria of each back-calculation standard concentration should be ±20% from the nominal value. The LOD for each bisphenol was determined from the product of standard deviations obtained for the observed concentration of spiked samples (0.15 ng/g, $n = 7$) by the Student's t test value, 3.14 (t-value at 99% confidence level, six degrees of freedom). On the other hand, LOQ determined for each bisphenol was ten times the standard deviation of the analyte concentration.[44] The lowest calibration standard with acceptable accuracy and precision was close to the calculated LOQ value for each analyte. The calculated LOQ was 0.09, 0.19, 0.18, 0.29, 0.28, 0.18, and 0.09 ng/g for BPA, BPE, BPAF, BPFL, BPC, BPZ, and BPS, respectively.

Accuracy and precision were quantified using QC samples at two concentration levels (0.19 ng/g or 0.78 ng/g and 80 ng/g) using six replicates in two different analytical batches on the same day (intraday) and two on consecutive days (interday). Freshly prepared calibration curves were used to analyze intra- and interday accuracy and precision, and the values were expressed as percent nominal (% nominal) and percent relative standard deviation (% RSD, should be ≤20%), respectively.

The percent recovery of BPs was also assessed at two concentration levels (0.19 ng/g or 0.78 ng/g and 80 ng/g) in six replicates by comparing the response of the analyte from the extracted formula samples with the postextracted samples spiked with analyte. As per SANTE guidelines, the acceptable limit of % recovery lies between 70% to 120%. A recovery outside this range is also acceptable provided they are consistent (RSD ≤ 20%), and the basis for this deviation is well established (e.g., due to analyte distribution in the partition step); however, the mean recovery should not be <30% or >140%.[43] Matrix effect was also determined by comparing the response of the postextraction spiked analyte sample with those of the standard solution ($n = 6$). Ion suppression or enhancement was calculated as % Matrix Effect (% ME) using the eq 1. % ME equals to 100% indicate no matrix effect, whereas >100% and <100% shows ion enhancement and suppression, respectively.[45]

$$\%\mathrm{ME} = \frac{\text{analyte response}_{(\text{post-extraction spiked matrix})}}{\text{analyte response}_{(\text{standard solution})}} \times 100 \quad (1)$$

Estimated Dietary Intake (EDI) of BPs in Infants (0−12 Months). Following validation, the developed analytical method was used for estimating the concentration of BPs in infant formula. The concentrations of BPs which were found equal to or greater than their respective LOQ in the infant formula were considered for estimating the daily intake. Estimated daily intake (EDI) of BPs was estimated for different age groups based on the volume of milk consumption and infant weight. The volume of milk consumption was measured by considering that 4.52 g of infant formula is required to prepare 30 mL of milk, according to the recommended intake by the manufacturers. For a one-week aged male infant, the average weight was 3.3 kg; for infants aged one month, 4.5 kg; for six-month, 7.9 kg; and for 12 months, 9.6 kg was considered. Whereas for one-week aged female infant, the average weight was 3.2 kg, for infants aged one month, 4.2 kg; for six-month, 7.3 kg; and for 12 months, 8.9 kg was considered. The milk consumption (mL) for 1 week, 1 month, 6 month, and 12 month old infant was 590, 642, 560, and 452 mL, respectively.[46] Equation 2 was used for calculating estimated daily intake (EDI) of BPs:

$$\text{estimated daily intake}\left(\text{ng/kg body}\frac{\text{weight}}{\text{day}}\right) = \frac{(\text{bisphenol concentration(ng/g)} \times \text{formula intake per day})}{\text{average body weight}} \quad (2)$$

The data were represented as mean and 95th percentile concentrations of BPs to depict the average and high exposure situations, respectively. Additionally, % frequency (% F) was also calculated to estimate the occurrence incidence of a particular bisphenol in 68 samples taken in the study. Further, hazard quotient (HQ) was calculated using eq 3. The HQ < 1 indicates that it is unlikely that the exposure population will experience the adverse health risk, and HQ > 1 indicates that there lies a possibility that the exposure population might experience adverse health effects.

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{TDI}} \quad (3)$$

where EDI is the estimated daily intake obtained from eq 2 and TDI is the tolerable daily intake of BPA (4 μg/kg of body weight per day). Since the TDI values for BPA analogues are not available, the TDI value of BPA was employed for estimating the HQ values for all of the BPs. In case the TDI value for all of the components is not available, the lowest available reference value can be used, assuming that all components with missing reference value are equally potent.[47]

RESULT AND DISCUSSION

Optimization of UPLC-MS/MS Conditions. To develop a sensitive, reproducible, and shorter analytical method, different columns, mobile phase compositions and strength of additives were tested. Of all the tested columns, best results were obtained using Phenonemex Kinetex C18 (100 × 2.1 mm, 1.7 μm) column in terms of peak shape and chromatographic run time. Next, mobile phase compositions were optimized and it was found that the peak shape of the analytes was better when acetonitrile was used as the organic phase. Inclusion of any acidity or basicity modifiers in the organic and aqueous mobile phase resulted in either poor peak shape or poor sensitivity of BPs. Therefore, acetonitrile and Milli-Q as the organic and aqueous mobile phase, respectively, in gradient mode were finalized which furnished a sharp and symmetrical peak shape of all of the BPs. Negative-ion detection mode was found to be suitable for all analytes and IS. The optimized MRM transitions for all of the BPs are represented in Table 1. The IS used for various analytes were IS-A for BPA; IS-AF for BPAF and BPFL; IS-E for BPC, BPE, and BPZ; and IS-S for BPS, respectively.

Optimization of the Sample Extraction Procedure. As BPs are omnipresent in the environment, special attention was given to the components that could interfere with the analysis. Solvents such as acetonitrile and Milli-Q water were tested before use for any contamination of BPs (Figure S1).

Infant formula is a complex matrix owing to the presence of high content of fats, fatty acids, and lipids.[48] Therefore, simultaneous extraction of polar as well as nonpolar BPs from the complex matrix of infant formula proved to be a challenging task. In this direction, QuEChERS technique was employed which involved the use of acetonitrile, anhydrous $MgSO_4$, and sodium chloride for the extraction of seven BPs in a single step. Significant efforts were made in the optimization of the extraction steps such as type of organic solvents

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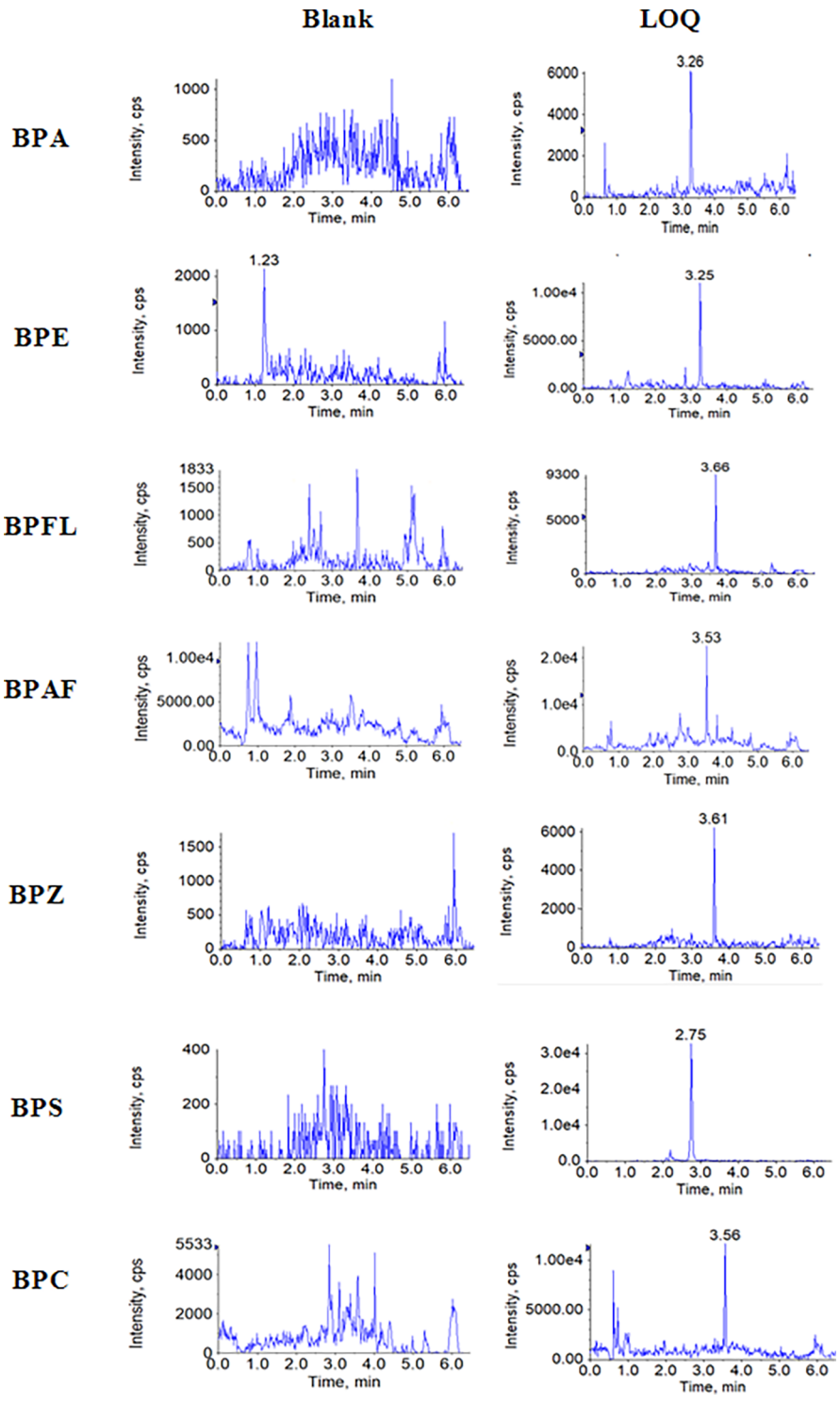

Figure 1. Representative MRM chromatograms of various bisphenol analogues in infant formula resulting from the analysis of (left to right) procedural blank sample and matrix spiked with analytes at limit of quantification (LOQ).

(methanol and acetonitrile), the composition of anhydrous $MgSO_4$ and sodium chloride and sonication time. Out of the organic solvents (acetonitrile and methanol) tested, acetonitrile gave satisfactory recovery of all the BPs. A composition consisting of acetonitrile, 2 g of anhydrous $MgSO_4$, and 1 g of sodium chloride proved suitable and efficient for the simultaneous extraction of all the BPs. In the quest to enhance the recovery of the BPs, a sonication step for 10 min was included which improved the recovery of all of the BPs. Next, the cleanup step was optimized wherein various sorbents, such as C18 and PSA and their different compositions, were tested. However, sample cleanup with these sorbents was not satisfactory (Table S1). As a result, samples were frozen for 30 min which resulted in the deposition of fatty materials on the walls of the tubes. Centrifuging the tubes separated the fatty components from the clean samples. To remove the particulate matter, the supernatant was passed through solvinert filter plates and centrifuged at 4000 rpm for 2 min.

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Table 2. Interday and Intraday Accuracy and Precision of BPs in Infant Formula

		interday (N = 6)			intraday (N = 12)		
analyte	conc. level	obs. conc.[a] (ng/g)	% accuracy[b]	% precision[c]	obs. conc.[a] (ng/g)	% accuracy[b]	% precision[c]
BPA	0.19	0.18 ± 0.01	95.35	7.55	0.18 ± 0.01	94.87	6.77
	80	84.33 ± 3.37	105.42	4.00	83.02 ± 3.76	103.8	4.53
BPAF	0.19	0.19 ± 0.01	99.12	3.03	0.19 ± 0.00	99.43	2.53
	80	83.68 ± 3.42	104.60	4.09	84.99 ± 5.33	106.24	6.27
BPC	0.78	0.86 ± 0.06	110.06	6.68	0.82 ± 0.06	104.85	7.84
	80	80.72 ± 4.02	100.89	4.99	81.02 ± 3.85	101.27	4.75
BPE	0.19	0.18 ± 0.01	96.40	5.81	0.18 ± 0.01	94.43	6.01
	80	88.67 ± 5.53	110.83	6.23	86.97 ± 4.72	108.71	5.42
BPFL	0.78	0.77 ± 0.04	98.48	5.84	0.75 ± 0.05	96.44	7.03
	80	84.78 ± 5.49	105.97	6.48	84.80 ± 4.51	106.01	5.31
BPS	0.19	0.19 ± 0.01	97.81	5.20	0.18 ± 0.02	96.10	10.18
	80	81.01 ± 0.67	101.26	0.83	82.78 ± 2.60	82.78	3.14
BPZ	0.19	0.18 ± 0.01	95.96	8.04	0.18 ± 0.02	95.96	8.33
	80	80.15 ± 7.87	100.19	9.82	81.83 ± 6.08	102.29	7.43

[a]Data are expressed as mean ± SD. [b]Calculated as (observed concentration/nominal concentration) ×100. [c]Expressed as % RSD (SD/mean) ×100.

Table 3. Recovery and Matrix Effect of BPs in Infant Formula at Two Concentration Levels

		recovery (N = 6)		matrix effect (N = 6)	
BPs	conc. level (ng/g)	% recovery[a]	% RSD[b]	% matrix effect[a]	% RSD[b]
Bisphenol-A	0.19	101.20 ± 7.05	6.96	98.48 ± 3.98	4.04
	80	103.91 ± 7.36	7.08	99.66 ± 3.72	3.73
Bisphenol-AF	0.19	91.73 ± 8.99	9.80	107.65 ± 9.05	8.41
	80	105.39 ± 8.46	8.03	100.74 ± 6.95	6.90
Bisphenol-C	0.78	105.74 ± 8.57	8.11	103.35 ± 6.50	6.29
	80	106.53 ± 3.92	3.68	98.69 ± 8.33	8.44
Bisphenol E	0.19	106.49 ± 6.61	6.20	100.75 ± 7.45	7.39
	80	99.25 ± 3.75	3.78	100.90 ± 7.09	7.02
Bisphenol-FL	0.78	102.45 ± 9.56	9.33	100.37 ± 5.96	5.94
	80	95.67 ± 6.00	6.28	100.14 ± 7.35	7.34
Bisphenol S	0.19	99.65 ± 3.59	3.60	100.84 ± 2.27	2.24
	80	96.53 ± 4.62	4.79	101.75 ± 4.40	4.32
Bisphenol-Z	0.19	97.83 ± 1.54	1.58	99.84 ± 2.05	2.05
	80	97.29 ± 2.78	2.86	102.11 ± 3.82	3.73

[a]Data are expressed as mean ± SD. [b]%RSD (SD/mean × 100).

Before submitting the filtered supernatant to UPLC-MS/MS analysis, 0.4 μL of 25% ammonia solution was added to 100 μL of supernatant (final concentration 0.1% ammonia in the sample) as it significantly improved the response and peak shape of all of the BPs, except BPS for which the response was drastically reduced. Thus, the optimized extraction and sample cleanup procedure furnished excellent recovery and peak shapes of all of the BPs.

Method Validation. For reliable quantification of BPs in powdered infant formula, the developed analytical method was validated as per SANTE Guidance Document.[43] Figure 1 shows the representative MRM chromatograms obtained from the analysis of the procedural blank sample and matrix spiked with analytes at LOQ. No significant interferences (<30% of the reporting limit (RL)) were observed at the retention time of the BPs. The LOQ was considered as the reporting limit (RL) for all of the BPs. Good linearity of the calibration curves of all of the BPs was displayed over the concentration range of 0.19−100 ng/g for BPA, BPAF, BPE, BPS, and BPZ and 0.78−100 ng/g for BPC and BPFL with a correlation coefficient of ≥0.98 in infant formula. The procedural blank sample, zero sample (blank with only IS), and ten calibration standards were taken in every batch of samples and the quantification of BPs in infant formula was conducted using the compound and IS ratio. The deviations of the back-calculated concentrations from the nominal values were well within the acceptable range. The LOD for BPs was in the range of 0.03−0.10 ng/g, whereas LOQ was in the range of 0.10−0.78 ng/g.

The interday and intraday accuracy and precision of BPs were well within the acceptable limits in infant formula at all of the examined concentrations and are shown in Table 2. This indicated that the developed analytical method was precise and accurate for the quantitative analysis of BPs in formula samples over the defined concentration range.

As shown in Table 3, the mean percent recoveries and percent matrix effect of BPs from the infant formula at various concentration levels were within the range of 91.73−106.53% and 98.48−103.35%, respectively, implying that an optimal analyte recovery with no significant matrix interference could be obtained from formula samples using the proposed extraction procedure.

Occurrence of BPs in Infant Formula. Infant formulas (N = 68) were collected from the Lucknow city of India and analyzed for the prevalence of various bisphenol analogues.

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Table 4. Concentration of Bisphenol Analogues in Infant Formula Collected from Lucknow City of India (n = 68)

infant formula	BPA	BPAF	BPC	BPE	BPFL	BPS	BPZ
mean (ng/g)	5.46	ND	ND	ND	ND	0.58	1.64
95th percentile (ng/g)	8.96	ND	ND	ND	ND	1.85	4.92
% detection frequency	76.47	ND	ND	ND	ND	27.94	13.23

Table 5. Estimated Daily Intake (EDI, ng/kg/b.w./day) of Bisphenol Analogue According to the Age of Male (M) and Female (F) Infants[a]

		1 week		1 month		6 months		12 months	
		F	M	F	M	F	M	F	M
BPA	mean	151.61	147.02	125.19	116.89	62.93	58.15	41.68	38.64
	HQ	0.038	0.037	0.031	0.029	0.016	0.015	0.010	0.010
	95th percentile	248.80	241.40	205.44	191.74	103.28	95.44	68.40	63.42
	HQ	0.062	0.060	0.051	0.048	0.026	0.024	0.017	0.016
BPS	mean	16.21	15.61	13.39	12.41	6.68	6.17	4.45	4.09
	HQ	0.004	0.004	0.003	0.003	0.002	0.002	0.001	0.001
	95th percentile	51.33	49.78	42.41	39.59	21.32	19.70	14.12	13.09
	HQ	0.013	0.012	0.011	0.010	0.005	0.005	0.004	0.003
BPZ	mean	45.54	44.16	37.60	35.09	18.09	17.46	12.52	11.60
	HQ	0.011	0.011	0.009	0.009	0.005	0.004	0.003	0.003
	95th percentile	136.53	132.29	112.80	105.28	56.71	52.40	37.56	34.82
	HQ	0.034	0.033	0.028	0.026	0.014	0.013	0.009	0.009

[a]Abbreviations: F, female infants; M, male infants; HQ, hazard quotient.

Out of seven BPs, six BPs including BPA, BPFL, BPAF, BPZ, BPC, and BPS were detected in at least one source of infant formula sample (Table 4). Most of the formula analyzed in this study contained detectable concentration of BPs. The most common bisphenol analogue detected in infant formula was BPA with % frequency of 76.47% (52/68) and mean concentration of 5.46 ng/g (95th percentile = 8.96 ng/g). This was followed by BPS and BPZ with detecting frequencies of 27.94% (19/68) and 13.29% (9/68), respectively. The mean concentration and 95th percentile values were 0.58 ng/g and 1.85 ng/g for BPS and 1.64 ng/g and 4.92 ng/g for BPZ, respectively. However, BPAF, BPFL, BPC and BPE were detected in none of the samples. No correlation could be established between the concentration of BPs detected with the type of package.

A review of the literature showed high variation among the results of BPA content in infant formula. The BPA concentration obtained in the present analysis were much lower than those reported from an analysis of ten infant formula collected from Italian and Spanish market (0.07−1.29 mg/kg).[28] Similarly, another report from Italy found BPA concentration in the range of 0.003−0.108 μg/g which was higher than that observed in the present analysis.[24] On the other hand, the findings of the present analysis were comparable with those reported from the USA (BPA mean level in infant formula was 0.97−1.24 ng/g).[49] In contrast, Sun et al. tested powdered infant formulas (N = 76) from China for the occurrence of BPA, however, no level was detected in any of the studied samples.[50] Literature information on bisphenol analogues is very few, only limited studies have considered their occurrence in infant formula.[51] One of the studies from Portugal has determined the occurrence of BPB in powdered infant formula (n = 7); however, it was detected in none of the samples.[52] Recently, a study determining the occurrence of BPA in infant formulas (N = 25) marketed in a city of Brazil has been reported. Their findings revealed that BPA was not present in any of the studied samples.[53] Various studies have been reported from India showing the presence of BPs in food, environment and human samples.[54−61] However, this is the first report determining the occurrence of BPs in infant milk powder (N = 68) from India. It should be noted that the main sources of contamination from BPs in infant formula are not known. The anticipated sources could be contamination during different stages of formula production (if the equipment/containers have epoxy coatings) and/or migration from packaging material of the storage containers having an epoxy coating and/or plastic components.[62,63] Besides, BPA can also come into the milk supply chain at primary production via consumption of contaminated animal feed and dairy farm environment.[62]

Owing to a structural similarity to BPA, various bisphenol analogues have shown comparable or even more potent endocrine-disrupting activity than BPA.[64] However, at present reference values, such as specific migration limit and/or TDI values, have been defined only for BPA and BPS.[65,66] Therefore, the occurrence of BPA and other bisphenol analogues in infant formula should be routinely examined to ensure the safety of the infants. Thorough investigations on other bisphenol analogues, such as constant biomonitoring and health hazard potential in human population, are urgently required so that regulatory guidelines on their occurrence in food, migration limits from packaging material, and tolerable daily intake (TDI) values can be properly defined.

Estimated Dietary Intake (EDI) of BPs in Infants (0−12 Months). Present is the first report providing exposure of seven BPs (BPA, BPAF, BPC, BPE, BPFL, BPS, and BPZ) to infants aged 0−12 months through the consumption of powdered infant formula used in India. Table 5 shows the mean and 95th percentile values of EDI of the BPs detected in the infant formula. The mean EDI values of total BPs lied in the range of 58.65−213.36 ng/kg b.w./day for female infants (0−12 months) and 54.33−206.79 ng/kg b.w./day (0−12 months) for male infants. Likewise, 95th percentile EDI values of total BPs lied in the range of 120.08−436.66 ng/kg b.w./day

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for female infants (0−12 months) and 111.33−423.47 ng/kg b.w./day for male infants (0−12 months). Among allof the BPs, BPA contributed to the majority of the total intake with mean EDI and 95th percentile values ranging from 41.68 to 151.6 ng/kg b.w./day and 68.40−248.80 ng/kg b.w./day, respectively, for 0−12 months aged female infants; and 38.64−147.02g/kg b.w./day and 63.42−241.40 ng/kg b.w./day for 0−12 months aged male infants, respectively. Among the four categorized groups, the exposure of BPA was highest in one-week-old infants (females and males) and one-month-old infants in comparison to 6- and 12-month infants. This might be due to the lower body weight of younger infants or higher consumption of milk by younger infants (one-week and one-month-old) in comparison to older infants (6−12-month old).[67] Followed by BPA, BPZ, and BPS contributed to the total intake. Apparently, no exposure was accounted for BPAF, BPFL, BPC, and BPE to 0−12-month-old female and male infants. The contribution (average value of female and male infants of age 0−12 months) of each bisphenol analogue to the total intake was in the following order: BPA (71.09%) > BPZ (21.35%) > BPS (7.55%). The mean EDI and 95th percentile values of total BPs obtained in the present study were rather lower than those reported earlier. For instance, an Expert Meeting conducted jointly by FAO/WHO concluded that the infants and children (0−6 months) were exposed to BPA through powdered formula (prepared as consumed) with a mean value of 2.0 μg/kg b.w./day and 95th percentile of 2.7 μg/kg b.w./day, respectively.[68] Likewise, the Scientific Committee of European Commission estimated that 1.6 and 0.8 μg/kg b.w./day was the dietary exposure of BPA for infants aged 0−4 months and 6−12-months through formula, respectively.[69] The dietary exposure of BPs was compared with the TDI value of BPA set by EFSA and US EPA as no safety threshold limit has been defined for other bisphenol analogues yet.

Considering all of the approaches, both mean and 95th percentile, the dietary exposure to total BPs for infants through infant formula estimated in the present study was approximately 1−2 orders of magnitude lower than the reference values of BPA set by EFSA (4 μg/kg b.w./day) and US EPA (50 μg/kg b.w./day), thus, posing no considerable risks to infants.[27,65,70,71] The HQ value obtained for BPA, BPS, and BPZ was found to be less than 1 which also indicated that the exposure of these bisphenols presents no apparent risk to infants through consumption of powdered formula. However, this is not a tranquillizing situation as BPA and its analogues even at low doses may produce serious health problems in infants.

CONCLUSION

This is the first report determining the occurrence of bisphenol analogues (BPA, BPAF, BPC, BPE, BPFL, BPS, and BPZ) in powdered infant formula from different Indian brands and estimating their dietary exposure to infants aged 0−12 months through the consumption of infant formula. For this, a simple and efficient LC-MS/MS method for the simultaneous determination of BPs (BPA, BPAF, BPC, BPE, BPFL, BPS, and BPZ) in Indian infant formula was developed and validated as per the SANTE Guidance Document. The linearity of all BPs was in the range of 0.19−100 ng/g for BPA, BPAF, BPE, BPS, and BPZ or 0.78−100 ng/g for BPC and BPFL with a correlation coefficient of ≥0.98. Mean percent recoveries and percent matrix effect of BPs was in the acceptable range. Following successful validation, the analytical method was employed to detect the occurrence of BPs in powdered infant formula (n = 68). The highest concentration was detected for BPA (mean = 5.46 ng/g; 95th percentile = 8.96 ng/g) followed by BPS and BPZ. However, the % frequency of BPs in infant formula was found highest for BPA (76.47%), respectively. BPAF, BPFL, BPC, and BPE were not detected in any of the studied samples. The mean EDI of total BPs was in the range of 58.65−213.36 ng/kg b.w. for female infants (0−12 months) and 54.33−206.79 ng/kg b.w. (0−12 months) for male infants, respectively. Among allof the BPs, BPA contributed to the majority of the total intake followed by BPZ and BPS. The EDI values of BPs exposure to infants were quite low when compared with the health-based reference values of BPA recommended by EFSA (TDI = 4 μg/kg/day). As bisphenol analogues have been reported to exhibit similar or higher toxic effects in comparison to BPA, their occurrence in infant formula raises serious concern over their use as a substitute for BPA in consumer products intended for infants.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00129.

MRM chromatograms of bisphenols in (A) acetonitrile blank, reagent blank, and mobile phase blank and (B) BPA, BPS, and BPZ peak detected in samples (Figure S1); optimization of (A) extraction and (B) cleanup procedure was done for the simultaneous quantification of bisphenols in infant formula (Table S1) (PDF)

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